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Original Article

Magnesium Hydride Attenuates Cognitive Impairment in a Rat Model of Vascular Dementia

Myeong Jin Lee[1], Ji Hye Lee[1], Do Kyung Kim[1], Nam Seob Lee[1], Young Gil Jeong[1], Ji Heun Jeong[1], Jong Ho Park[2], Yung Choon Yoo[3], Seung Yun Han[1]

[1]*Department of Anatomy, College of Medicine, Konyang University*
[2]*Department of Medicinal Materials, College of Medical Engineering, Konyang University*
[3]*Department of Microbiology, College of Medicine, Konyang University*

Abstract : Reactive oxygen species (ROS)-mediated oxidative stress plays a key role in the pathogenesis of central nervous system diseases, including vascular dementia (VaD). Thus, scientific attention has been given to the uptake of molecular hydrogen (H_2), a powerful ROS scavenger that is abundant in nature, as a potential therapeutic candidate. Among the methods to supply H_2, we selected an oral supplement of magnesium hydride (MgH_2) and investigated its therapeutic role in cognitive impairment and hippocampal neuronal death associated with VaD. Sprague Dawley rats were randomly divided into 4 groups (*n* of each = 8) and subjected to different conditions: SO, a group with vehicle and sham-operation; VEH, a group with a vehicle and 2VO/H (2 vessel occlusion and hypovolemia, used as a surgical model of VaD); MH-L, a group with low dose (5 mg/kg) of MgH_2 and 2VO/H; and MH-H, a group with high dose (15 mg/kg) of MgH_2 and 2VO/H. MgH_2 or vehicle was administered via an intragastric route for 14 days before the operation. Subsequently, the memory performances of rats were tested using three behavior tests, *i.e.*, Y-maze-, Barnes maze-, and passive avoidance tests (PAT). On postoperative day 8, the number of viable neurons in the hippocampal Cornu Ammonis (CA) 1 region was measured. The results of behavioral tests revealed that memory performance was significantly hampered in the VEH group when compared with the SO group; however, the extent of the impairment was markedly diminished in the MH-L and MH-H groups. While the number of pyramidal neurons in hippocampal CA1 was largely reduced in the VEH group when compared with the SO group, this reduction was significantly attenuated in the MH-L and MH-H groups. The effects of MgH_2 were dose-dependent in PAT and histologic experiments. These results suggest that MgH_2 supplementation can attenuate cognitive impairment and hippocampal neuronal death associated with VaD.

Keywords : Magnesium hydride, Vascular dementia, 2VO/H, Hippocampal neuron

INTRODUCTION

Vascular dementia (VaD), the second most common type of dementia after Alzheimer's dementia, triggers cognitive impairment (dementia) attributable to cerebrovascular accident (CVD) [1]. When blood supply to the

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Correspondence to: Seung Yun Han (Department of Anatomy, College of Medicine, Konyang University, Daejeon 35365, Republic of Korea)
E-mail: jjzzy@konyang.ac.kr

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brain is interrupted after CVD, cellular components of the central nervous system (CNS) are deprived of vital oxygen and glucose, causing damage to responsible CNS subfields, such as the hippocampus, associated with learning and memory [2].

There is evidence that ischemic insults finally cause VaD-associated neuronal death through intracellular accumulation of reactive oxygen species (ROS) [3]. In fact, under ischemic insults, deprived supplementation of oxygen and glucose elicits an increase in glutamate release from the axon terminals of presynaptic neurons, which in turn causes excessive calcium entry into dendritic spines of postsynaptic neurons [4]. Calcium then triggers ROS overproduction by affecting mitochondrial dysfunction [5]. Since excitotoxicity is inevitably linked to ROS accumulation, antioxidant treatment is likely to be an appropriate strategy for protecting against VaD [6].

Molecular hydrogen (H_2) is an effective and bio-safe molecule with a wide mode of action for treating various ROS-related diseases, including VaD [7]. Although the vast majority of potential mechanisms of action remain to be elucidated, it has been revealed that H_2 exerts its beneficial effects through antioxidant, anti-inflammatory, and antiapoptotic effects, thus providing cytoprotection [8]. The protective effects of H_2 on pathological conditions have been investigated using animal models of fibrotic heart disease [9], hepatic fibrosis [10], cerebral ischemia [11], radiation injury [12], and diabetes [13], in which intracellular accumulation of ROS is essentially involved in their pathogenesis.

There are several methods to administer H_2, including inhaling gaseous hydrogen, drinking H_2-rich water, and injecting H_2-rich saline. Drinking H_2-rich water attenuated stress-induced memory impairments in mice by reducing oxidative stress [14]. In addition, injection of H_2-rich saline enhanced memory function in rats with amyloid-β-triggered dementia by reducing oxidative stress [15]. Moreover, inhalation of gaseous H_2 during resuscitation rescued ischemic neuronal damage in a rat model of cardiac arrest [16]. An alternative to supplying H_2 that has gained scientific attention, involves the reaction of metal hydrides with water. Although research trials on organisms are scarce, with this method, hydrogen can be directly generated onsite by the hydrolysis reaction of metal hydrides, *e.g.*, magnesium hydride (MgH_2), in a moist environment such as the alimentary tract [17]. The most important advantages of this reaction are that (i) the weight yield of the released hydrogen is high when water is included; in detail, 1 g of MgH_2 produces 1820 cc of H_2, (ii) the byproduct $Mg(OH)_2$ is biocompatible, and (iii) the low cost of MgH_2 [18-20]. Although pure MgH_2 hydrolysis occurs extremely slowly, the kinetics and H_2 yield can be strongly accelerated in the presence of acidic pH. As the pH of gastric acid is 1.5～3.5, we hypothesized that H_2 can be effectively generated from MgH_2 *in situ*, i.e., in the mammalian stomach lumen. In this study, we examined whether intragastric administration of MgH_2 could suppress memory impairment and hippocampal neuronal death in a rat model of VaD.

MATERIALS AND METHODS

Preparation of MgH_2

MgH_2 was synthesized and kindly gifted by Solco Biomedical (Pyeong-Taek, Korea). Briefly, the two chemicals, magnesium (24.3 in atomic weight, 99.99% purity, Metal player, Incheon, Korea) and hydrogen (2 in atomic weight, 99.99% purity, Deokyang, Ulsan, Korea) were reacted in custom-made equipment (Reactor System, Solco biomedical) under high temperature (351～370°C) and high pressure (0.96 Mpa) for 90 h to yield 7.6 wt% ($2/24.3+2\times100$) of H_2 as described previously (#US8758643B2).

Measurement of the dissolved H_2 concentration

Five-hundred milligrams of MgH_2 powder was dissolved in 200 mL normal saline, and the pH adjusted to 2.5, to mimic intragastric conditions, while continuous stirring at 37°C was done. The H_2 concentration of the mixture was measured using a hydrogen concentration measuring instrument (KM2100 DH, Kyoei Denshi Kenkyuujo, Japan) at pre-determined times.

Animals

A total of 32 male Sprague Dawley rats (8 weeks, 200～250 g) were purchased from Samtako (Osan, Korea). The rats were housed in an environmentally controlled room at constant temperature (21～23°C) and relative humidity (40～60%) under a 12-h light/dark cycle. All rats had free access to water and food. Experiments were conducted in accordance with the 'Guide for the Care and Use of Laboratory Animals' (National Institutes of Health publi-

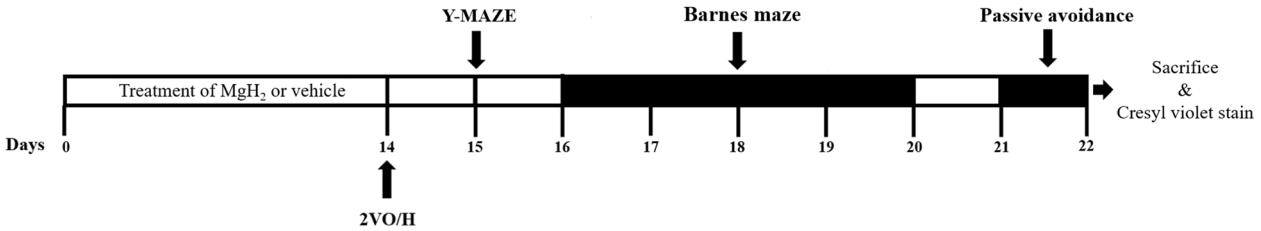

Fig. 1. Timeline of experimental protocols. A total of 32 rats ($n=8$ per group) were pretreated with the vehicle or MgH_2 for 14 days and subsequently underwent 2VO/H or sham-operation, yielding 4 groups as follows: SO, a group treated with vehicle and sham-operation; VEH, a group treated with vehicle and 2VO/H; MH-L, a group treated with 5 mg/kg MgH_2 and 2VO/H; and MH-H, a group treated with 15 mg/kg of MgH_2 and 2VO/H. Using these rats, three different behavioral tests, *i.e*, the Y-maze, Barnes maze, and passive avoidance tests, were conducted at the indicated times. For histological analyses, all rats were sacrificed immediately after finishing the three behavioral tests.

cation, 8th Edition, 2011) [21]. The animal experiments in this study were approved by the Institutional Animal Care and Use Committee (approved protocol number: P-18-14-A-01) of Konyang University (Daejeon, Korea).

Experimental design

All rats were randomly assigned to 4 groups ($n=8$ per group) and then treated differently as follows: SO, a group treated with normal saline (0.9% *w/v* of sodium chloride) used as a vehicle and underwent sham-operation; VEH, a group treated with a vehicle and underwent 2 vessel occlusion and hypovolemia operation (2VO/H); MH-L, a group treated with a low dose (5 mg/kg) of MgH_2 and 2VO/H; and MH-H, a group treated with a high dose (15 mg/kg) of MgH_2 and 2VO/H. MgH_2, dissolved in a final 1 mL volume, or vehicle was administered daily via the intragastric route for 14 days before the operation. The experimental design adopted in this study is illustrated in Fig. 1.

Two vessel occlusion and hypovolemia (2VO/H) operation

To establish an animal model of VaD, 2VO/H was employed using the method previously described by Smith et al. [22] with some modifications. For this, anesthesia was achieved with 5% isoflurane in 70% N_2O and 30% O_2 and maintained during the operation at a level of 1.5~2% isoflurane under spontaneous respiration. Throughout the operation, the rectal temperature was controlled at 37°C with a heating pad. The left femoral artery was exposed and catheterized with a PE-50 catheter to allow future withdrawal of blood to cause hypovolemia. The right jugular vein was isolated and injected with 500 U/kg heparin dissolved in 100 U/mL with 0.9% saline. After exposing the bilateral common carotid arteries (CCAs), these two vessels were permanently ligated with a 4-0 nylon suture. Next, blood was gradually withdrawn from the femoral artery via a catheter to achieve a reduction of cerebral blood flow (CBF). When the relative CBF (rCBF) value, measured by laser Doppler flowmetry (Periflux5000; PerimedAB, Järfälla-Stockholm, Sweden), was approximately $<20\%$ of baseline, withdrawal of blood was stopped for 10 min to maintain an ischemic period. Throughout this period, the syringe filled with pulled blood was kept in a shaking water bath at 37°C to prevent coagulation. Subsequently, the blood was reinfused, rapidly returning the CBF to baseline. After reaching a plateau, the animals regained consciousness and were maintained in their home cage until further experimentation.

Y-maze test

The Y-maze test was employed as the first tool to assess memory performance. For this, the rats were initially introduced to the center of the matte black plastic maze with three arms at 120-degree intervals, which were 50 cm long, 15 cm wide, and 30 cm high. The sequence and number of arm entries were monitored for an 8-min period. To calculate the percentage of spontaneous alternation, the following formula was used: spontaneous alternation (%) = [(number of alternations)/(total number of arm entries-2)] × 100. The number of total arm entries also served as an indicator of locomotor activity. After each rat was tested, the maze was cleaned using 70% ethanol.

Barnes maze test

The Barnes maze test was employed as the second tool

to assess memory performance. For this, a 100-cm-high circular platform with a diameter of 122 cm was used. There are 20 holes located around the perimeter with a black escape box (20 × 15 × 12 cm) placed under one of these. The task was divided into two stages: stage 1, training trials on postoperative days (POD) 2, 3, 4, and 5; and stage 2, a probe test on POD 6. During the training trials, the animal was placed on the table and given 120 s to find and enter the escape box, with bright lights as aversive stimuli. Each animal performed one trial per day during this stage. On the probe test day, the escape box was removed, and the time spent in the quadrant where the escape box was originally located was recorded. The explorative behavior of individual rats during stages 1 and 2 was recorded and analyzed with the aid of a video camera connected to an EthoVision XT9 system (Noldus, Wageningen, Netherlands). After each rat was tested, the circular platform and escape box were cleaned using 70% ethanol.

Passive avoidance test

The passive avoidance test was employed as the third tool to assess memory performance. The test apparatus was equipped with two chambers, *i.e.*, an illuminated chamber and a dark chamber, each measuring 25 × 20 × 25 cm. A lamp (50 W) was used as the source of light in the illuminated chamber. Each test involved two separate sessions: a trial session and a test session. During the trial session (POD 7), the rats were initially placed in the illuminated chamber. The "trial latency" time, once the rat had entered the dark compartment, was measured using a stopwatch. This measurement was triplicated and at the last entry of the rat to the dark chamber, an electrical shock (0.5 mA) for 3 s was delivered through stainless steel rods. A test session was performed 24 h following the trial session, and the "escape latency" times to re-enter the dark chamber were measured up to 180 s. After each rat was tested, the walls of the chambers were cleaned using 70% ethanol.

Cresyl violet staining

On POD 8, rats were transcardially perfused with 4% paraformaldehyde (PFA). Their brains were isolated and post-fixed with 4% PFA, dehydrated with a graded ethanol series, embedded in paraffin, and serially sectioned at 5-μm thickness using a microtome (RM2255, Leica, Nussloch, Germany). The two slides randomly selected from the hippocampus-bearing tissue collections per rat were de-paraffinized in xylene, hydrated in a decreasing ethanol gradient series, and washed twice in distilled water. The slides were then stained with a 0.1% Cresyl violet solution (Sigma-Aldrich, St. Louis, MO, USA). Each hippocampal Cornu Ammonis (CA) 1 region was photographed at ×400 magnification using a digital camera connected to a light microscope (DM4, Leica). The number of intact neurons localized in the CA1 subfield at 300 μm in width was counted and averaged in the photographs. At this time, only neurons with clear nuclei and large cell bodies were considered morphologically intact.

Statistical analyses

All data are presented as the mean ± standard error of the mean (SEM). Data comparison between groups was performed using one-way ANOVA in GraphPad (GraphPad Prism 5; GraphPad Software, Inc., San Diego, CA, USA). $P < 0.05$ indicated a statistically significant difference.

RESULTS

MgH_2 continuously generated H_2 in an acidic aqueous environment

MgH_2 was first tested for its time-dependent H_2-generating effects under conditions mimicking the stomach lumen. As shown in Fig. 2, 500 mg MgH_2 powder could generate 2000 ppb H_2 immediately after dissolution in normal saline (pH 2.5) at physiological temperature (37°C). Approximately half of the initial amount of H_2 was released from MgH_2 by 7.5 days, and the complete depletion of releasable H_2 was observed by 21 days. These observations indicate that MgH_2 can continuously generate H_2 in an acidic environment such as the intragastric lumen. After confirming sustained generation of H_2 from MgH_2, rats were treated with MgH_2 or normal saline as a vehicle using a daily regimen for 14 days before 2VO/H operation.

MgH_2 attenuated memory impairment in VaD rats

Thereafter, we investigated whether intragastric admin-

istration of MgH_2 can prevent memory impairment, an essential feature in VaD patients. For this, we assessed the effects of chronic intake of MgH_2 on VaD-associated memory impairment using rats who underwent 2VO/H, a surgical model of VaD. Three different behavioral analyses, that is, Y-maze, Barnes maze, and passive avoidance tests, were employed to assess memory employment in the rat groups. The results from the Y-maze test indicated that the VEH group showed remarkable impairments in memory function, as shown by spontaneous alternation ($p^{***} < 0.001$ vs. CTRL; Fig. 3A). However, both the MH-L and MH-H groups showed a significant increase in spontaneous alternation behavior when compared with the VEH group ($p^{\#} < 0.05$ and $p^{\#\#} < 0.01$ vs. VEH, respectively), although not dose-dependent. Neither 2VO/H nor MgH_2 treatment significantly affected locomotion, as demonstrated by total arm entry (Fig. 3B). The results from the Barnes maze test demonstrated that all the groups spent similar amounts of time exploring the escape box on the first day of the training trials (Fig. 4A-F). However, on the 2nd, 3rd, and 4th day of training trials, the VEH group spent a longer time and moved a longer total distance to find the escape box when compared with the SO group ($p^{***} < 0.001$ vs. SO). Interestingly, the escape latency and the moving distance were significantly decreased in the MH-L and MH-H groups when compared with the VEH group at all times in the training trials except on the 1st day ($p^{\#} < 0.05$ and $p^{\S} < 0.05$ vs. VEH, respectively). Neither 2VO/H nor MgH_2 treatments significantly affected locomotion, as shown in the velocity plots (Fig. 4G). During the probe test, the VEH group spent less time in the target quadrant where the escape box was formerly located ($p^{**} < 0.01$ vs. SO), suggesting that 2VO/H triggered memory deficits in rats (Fig. 5A and 5B). Interestingly, this deficit was prevented in the MH-L and MH-H groups, although dose-independently. Finally, the effect of MgH_2 on AD-associated memory impairment was assessed using the passive avoidance test. The results showed that the VEH group developed a significant impairment in memory retention when compared with the SO group, as evidenced by the shorter escape latencies ($p^{**} < 0.01$ vs. SO; Fig. 6A) in the test session. However, the escape latencies of the MH-L and MH-H groups were significantly longer than those of the VEH group ($p^{\#} < 0.05$ and $p^{\#\#} < 0.01$, respectively), and these changes were dose-dependent ($p < 0.05$). As shown in Fig. 6B, neither 2VO/H nor MgH_2 affected the trial latencies assessed

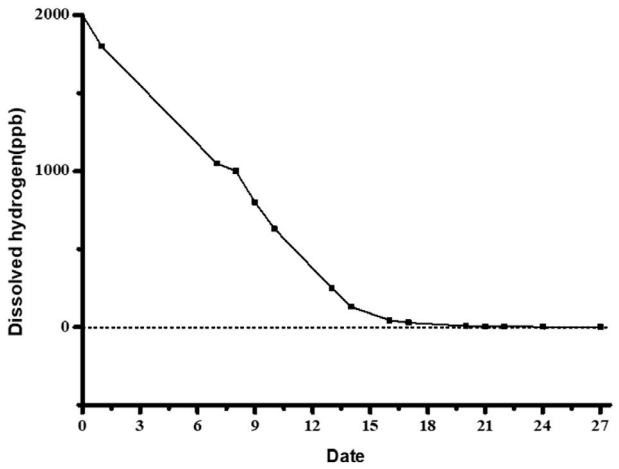

Fig. 2. MgH_2 dissolution curve. The vehicle used was acidic normal saline (0.9% *w/v* sodium chloride). 500 mg MgH_2 powder was dissolved in 200 mL normal saline and the pH adjusted to 2.5, thus mimicking the gastric lumen. H_2 concentration was measured using a hydrogen meter at pre-determined times.

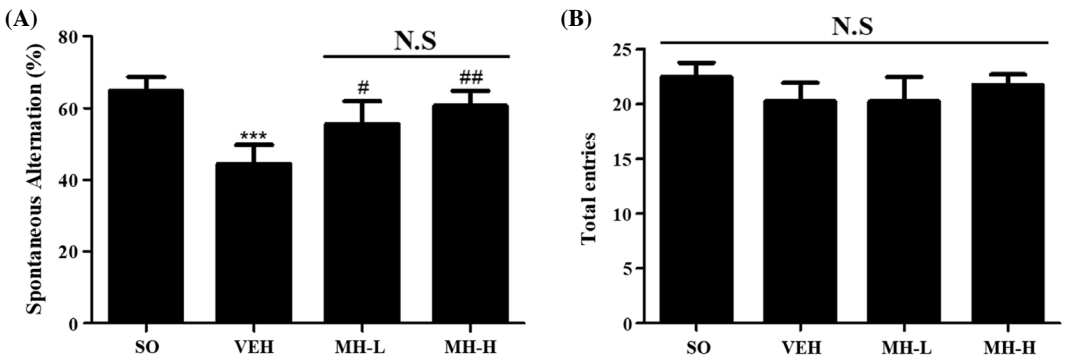

Fig. 3. Effects of MgH_2 on spontaneous alternation in rats with vascular dementia. These were tested using the Y-maze test. The average spontaneous alternation rate (A) and the number of total arm entries for >8 min (B) were measured in rats of different groups. Values are presented as the mean ± SEM ($p^{***} < 0.001$ vs. SO; $p^{\#} < 0.05$ and $p^{\#\#} < 0.01$ vs. VEH; N.S, no significance).

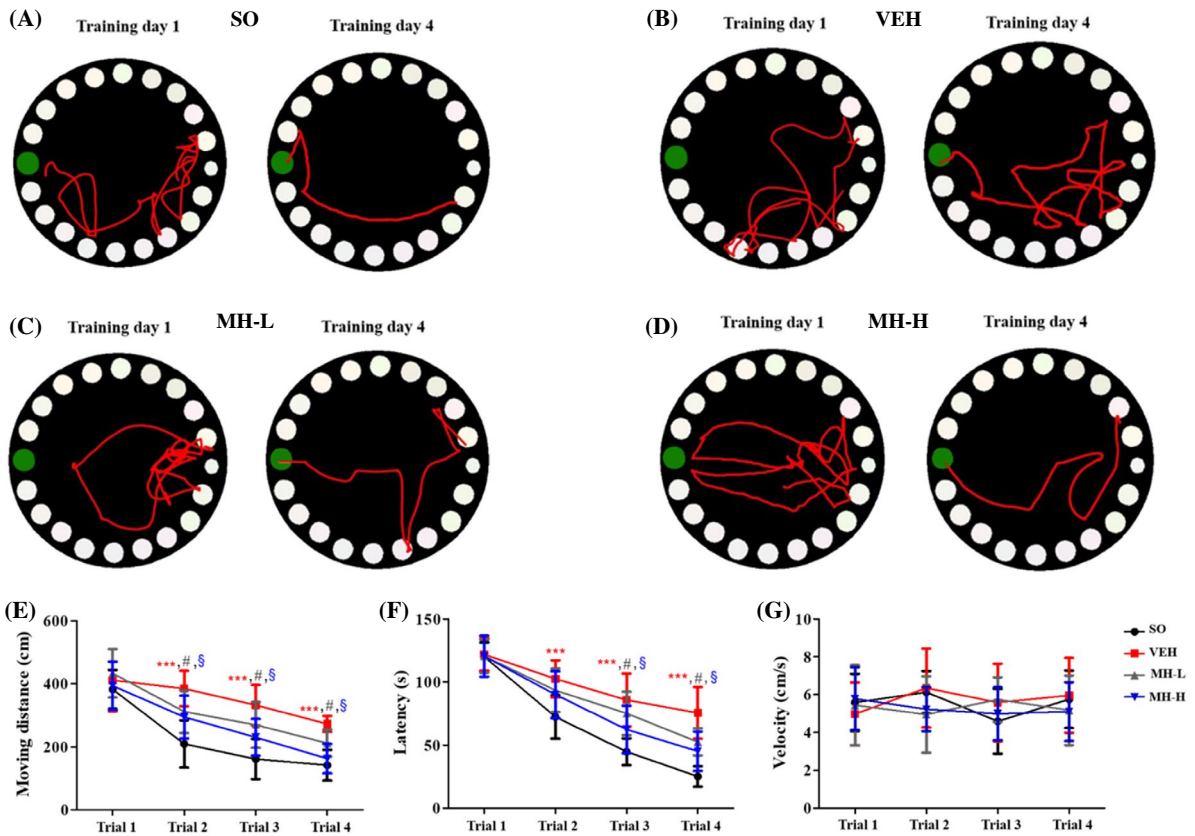

Fig. 4. The effects of MgH_2 on escape latency in rats with vascular dementia. These were tested using the Barnes maze training trial. Representative tracking plots of SO (A), VEH (B), MH-L (C), and MH-H groups (D) on the 1st and 4th day of the Barnes maze training trial. Moving distance (E), escape latency (F), and escape velocity (G) for up to 120 s were recorded. Values are presented as the mean ± SEM ($p^{***} < 0.001$ vs. SO; $p^{\#} < 0.05$, MH-L vs. VEH; $p^{\S} < 0.05$, MH-H vs. VEH).

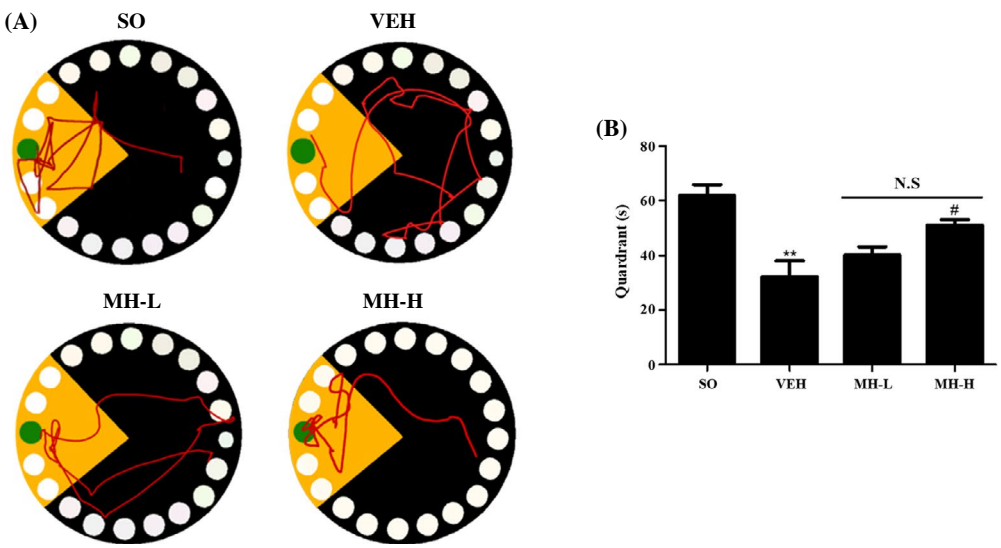

Fig. 5. The effects of MgH_2 on time spent in the target quadrant. These were tested using the Barnes maze probe test. Representative tracking plots of SO, VEH, MH-L, and MH-H groups (A) are shown. The time spent in the target quadrant during the probe test was recorded and statistically analyzed. Values are presented as the mean ± SEM ($p^{**} < 0.01$ vs. SO; $p^{\#} < 0.05$ vs. VEH; N.S, no significance).

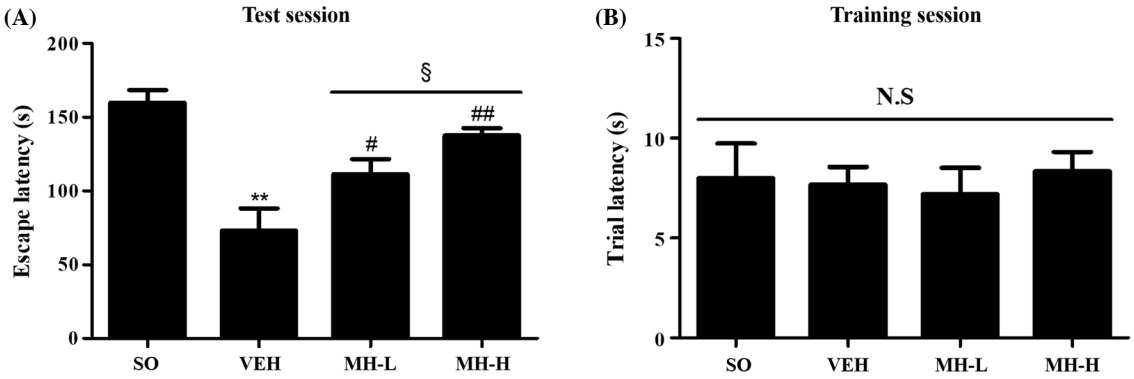

Fig. 6. The effects of MgH_2 on escape latency in rats with vascular dementia. These were tested using the passive avoidance test. The escape latencies (A) and the trial latencies (B) to enter the dark chamber during the test and trial sessions, respectively, are shown. Values are presented as the mean ± SEM ($p^{**} < 0.01$ vs. SO; $p^{\#} < 0.05$ and $p^{\#\#} < 0.01$ vs. VEH; $p^{\S} < 0.05$ vs. MH-L; N.S, no significance).

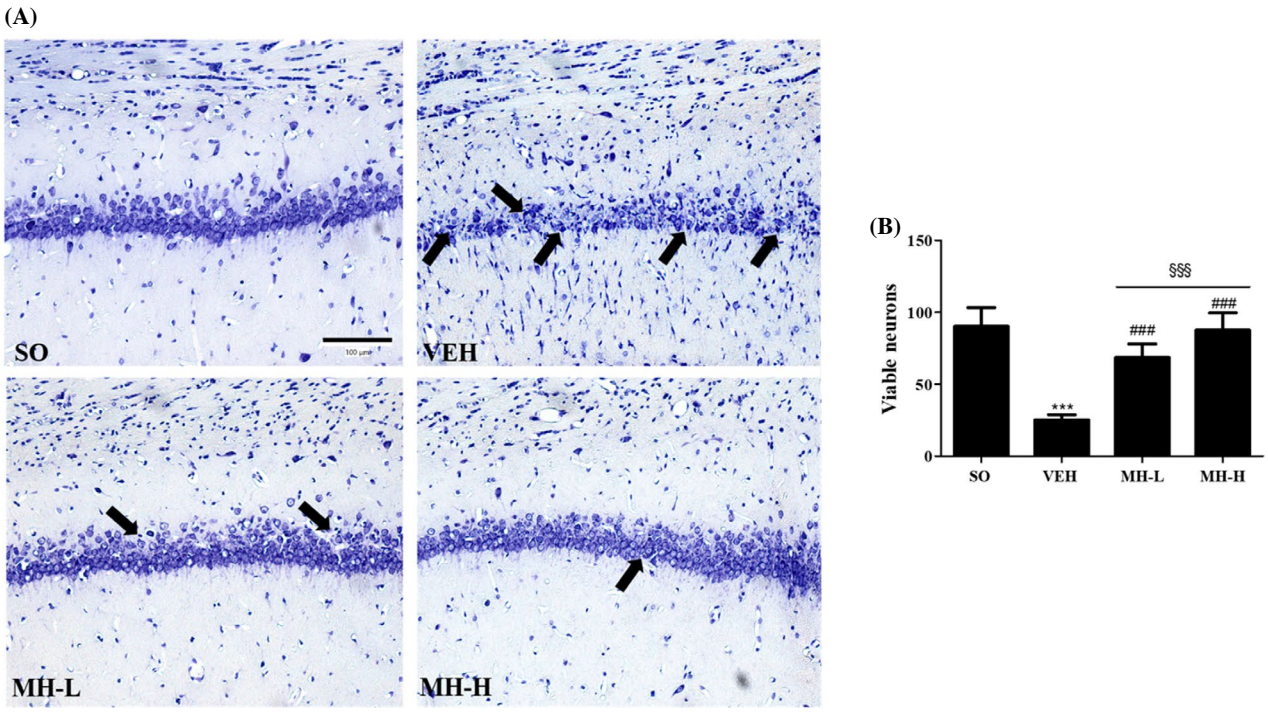

Fig. 7. The effects of MgH_2 on hippocampal neuronal survival. These were analyzed in rats with vascular dementia on postoperative day 8. A representative Cresyl violet-stained image (A) and the graph showing the average number of surviving neurons (B) in the hippocampal CA1 region. The region of interest was within 300 μm in the hippocampal CA1 region. Neurons undergoing apoptosis characterized by pyknotic nuclei and shrunken cytoplasm are indicated with black arrows. Values are presented as the mean ± SEM ($p^{***} < 0.001$ vs. SO; $p^{\#\#\#} < 0.001$ vs. VEH; $p^{\S\S\S} < 0.001$ vs. MH-L).

during the training session, indicating that seeking behavior or sensitivity to light was not affected by 2VO/H and MgH_2. Together, these results suggest that MgH_2 significantly ameliorated spatial learning and memory deficits in VaD rats.

MgH_2 attenuates deteriorations in hippocampal structures in VaD rats

Next, we investigated whether MgH_2 can prevent morphological deterioration in the hippocampal structure, an essential feature in either 2VO/H-induced VD rats or VD

patients. For this, using Cresyl-violet stain, we assessed the effects of 14 days of pretreatment with MgH_2 on 2VO/H-induced loss of pyramidal neurons in the hippocampal CA1 region. On POD 8, the VEH group showed a dramatic decrease in the number of hippocampal CA1 neurons when compared with that of the SO group, as shown in Fig. 7A and 7B ($p^{***} < 0.001$ vs. SO). In the VEH group, in addition to the loss of neuron number, the neurons undergoing apoptosis, characterized by pyknotic nuclei and shrunken cytoplasm, were abundant in the stratum pyramidale (SP) of the hippocampal CA1 region (black arrows in Fig. 7A). However, the number of surviving neurons was significantly increased in the MH-L and MH-H groups ($p^{\#\#\#} < 0.001$ vs. VEH), and this change was dose-dependent ($p^{\delta\delta\delta} < 0.001$). Collectively, these results suggest that intragastric administration of MgH_2 could prevent VaD-associated hippocampal neuronal loss.

DISCUSSION

Molecular hydrogen (H_2), which is nonpolar, small, and electrically neutral, can rapidly penetrate all cell membranes; as such, it can easily cross the blood-brain barrier. In 2007, Ohsawa et al. reported that gaseous H_2 acts as a therapeutic antioxidant in cells by selectively scavenging ROS and reactive nitrous species (RNS), such as the hydroxyl radical ($^{\bullet}OH$) and peroxynitrite ($ONOO^-$), thus conferring cytoprotection against oxidative damage [23]. Since this strong therapeutic effect of H_2 on a rodent model of cerebral ischemia was reported, multiple studies showed that several experimental models of pathology can benefit from H_2. An increasing number of studies revealed the therapeutic effects of H_2 in various CNS disease models, and that it is due to its anti-oxidative, anti-apoptotic, and anti-inflammatory activities [24]. However, the potential molecular mechanisms are still unclear, and some results remain controversial, thus requiring further research. Moreover, since the current findings are mainly based on animal experiments, whether these findings can translate to humans remain unanswered. Therefore, further human studies are needed to validate the findings seen in rodents. Nevertheless, the advantages of H_2 have provided important means and optimistic prospects for preventing CNS diseases.

The right amount of hydrogen supplementation might have certain benefits for treating various pathologies, including VaD. H_2-rich water has received widespread attention in the international market in recent years. However, the widespread use of H_2-rich water is restricted by the low solubility of H_2 [25]. The hydrogen content in H_2-rich water is generally approximately 0.1∼1 ppm, with the highest concentration known to be approximately 5 ppm [26]. To overcome the issue of low H_2 concentration, we used MgH_2 as the H_2 donor in this study. When compared with previous reports that used H_2-rich water as an H_2 donor, the present trial has the following beneficial effects: 1) a supplement of magnesium and hydrogen preparation can simultaneously supplement magnesium and H_2, which can neutralize ROS and RNS; 2) unlike H_2-rich water, limited by the low solubility of hydrogen, the amount of H_2 supplemented by our preparation is greatly improved in the acidic environment of the stomach; 3) the reaction metabolites (Mg^{2+}, $2OH^-$, and $2H_2$) have no toxic side effects or adverse reactions and, therefore, are safe and reliable; and 4) since MgH_2 is a powder, its production cost is low; therefore, it is suitable for large-scale production and storage.

Using this formulation, H_2 could be supplied in a sustainable manner; therefore, we tested its effects on an *in vivo* experimental model of VaD achieved surgically, *i.e.*, 2VO/H. Traditionally, artificial interruption of all four vessels that supply the brain, both CCAs and vertebral arteries, the so-called 4 vessel occlusion technique (4-VO), has been widely used to produce a rat model of VaD [27]. While both CCA occlusions can be accomplished using a minimally invasive ventral neck incision and application of aneurysm clips for the desired period, the occlusion of both vertebral arteries can be technically difficult, as they are located within the transverse foramina of the 2nd cervical vertebra, which is hardly visible under gross inspection [28]. In contrast with 4-VO, both CCAs occlusion coupled with systemic reduction of mean arterial blood pressure leading to a significant decrease in CBF, i.e., the so-called 2VO/H model, can produce ischemia throughout the forebrain, resulting in a pattern of brain damage closely mimicking CVA seen in VaD. In our preliminary experiments and from the results obtained in this study, POD 8 represents the optimal time at which CA1 hippocampal neuronal death can be quantified using Cresyl violet staining. Thus, all remaining cells at POD 8 can be assumed to be viable cells, thus providing an index of brain damage or rescue after certain interventions.

This study provides evidence that MgH_2 supplementa-

tion can exert anti-VaD effects, attenuating memory deficit and hippocampal neuronal death, the two essential features of VaD. However, the major limitations of this study are still remained. First, we could not rule out the possibility of involvement of magnesium effect on MgH_2-triggered anti-VaD effect. In fact, a few studies clearly demonstrated that magnesium supplement attenuated the VaD-associated symptoms [29] and risks [30] in human. Thus, further studies for dissecting out of the possible involvement of magnesium from MgH_2-induced protection against VaD is remained to be fulfilled. Second, this study lacks detailed explanations regarding mechanisms underlying MgH_2-mediated anti-VaD effects *in vivo*. Considering that VaD involves complex pathological cascades, including a series of molecular and cellular events such as apoptosis, neuroinflammation [31], and mitochondrial dysfunction other than ROS-associated oxidative damage [32], more advanced and well-designed studies should be performed in the future to provide a more detailed explanation about the specific mechanisms underlying MgH_2-mediated protection against VaD.

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